Identification and Inhibition of C-Terminal ATP-Binding Sites of Hsp90α Through Design of Novel Derrubone Analogues as a Plausible Approach Towards Alzheimer's Disease Treatment

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Certificate

This is to certify that the thesis entitled "**Identification and Inhibition of C-terminal ATP-Binding Sites of Hsp90α Through Design of Novel Derrubone Analogue as a Plausible Approach Towards Alzheimer's Disease Treatment**" by **Shumaila Khalid (212BM2012)** submitted to the National Institute of Technology, Rourkela for the partial fulfillment of the Degree of Master of Technology is a record of bonafide research work, carried out by her in the Department of Biotechnology and Medical Engineering under my supervision and guidance. To the best of my knowledge, the matter embodied in the thesis has not been submitted to any other University/ Institute for the award of any Degree or Diploma.

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Real life is not always going to be perfect or go the way we want it to, but the recurring acknowledgement of what is works for us in our lives can help us not only to be thankful but also surmount our difficulties.

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(SHUMAILA KHALID)

CONTENTS

ABSTRACT

Alzhiemer's disease (AD) is an age-old known neurodegenerative disorder caused by the formation of insoluble plaques within and around the neurons of the central nervous system resulting in lack of emotional and memory response of an individual. Till date there have been a variety drugs made available in the market which help combat or regress the pace of the symptoms of the disease, but none so-ever formulated commercially to treat the cause of the disease.

One of the most significant causative factors of the disease progression is the failure of the heat shock protein Hsp-90 to adequately refold the insoluble *Tau* protein tangles formed within the neuronal cytoplasm, and concurrently not being able to trigger the proteolytic degradation of the same. A variety of drug molecules have been hitherto designed and tested for satisfactory insilico and invitro inhibition of the Hsp90 protein complex, rendering the cellular system to switch to proteasomal degradation pathway towards the tangled *Tau* elimination. Inhibition of C-Terminal of Hsp90α by blocking its ATP binding sites might be used as an effective therapeutic approach for the treatment of AD via degradation of *Tau* proteins.

In our present work, it was observed that Leu 666, Leu 666 and Leu 694 could possibly be the ATP binding sites of Hsp90α CTD. It was also known from the experimental results that among the existing drugs, Derrubone showed the best binding affinity (-7.53 kcal/mol) for Hsp90α CTD.

Derrubone, which had proved its mark as the best among inhibitory molecules, has been structurally modified resulting in molecular analogues that have basic molecular skeleton of the original drug but serve improved ADME and inhibitory properties as against the same. Additionally, two previously experimentally synthesized Derrubone analogues developed by Blagg *et. al.* have been analyzed for validation of our recognized ATP binding sites and

compared with the newly designed analogues for their inhibitory properties at the recently recognized ATP-binding sites of the Hsp-90 molecule. Among the 30 analogues designed and tested insilico for various properties such as lipophilicity (AlogP), inhibitory constants (k_i), binding energy, carcinogenicity, mutagenicity, biodegradability using software viz. Discovery Studio 4.0, AutoDock 4.0 and other online tools; Analogue 26 consisting of ether substitution at the 5^{th} position of the isoflavon backbone proved a better drug as compared to Derrubone and the analogues developed at the laboratory of Blagg *et. al,* with a binding energy of -10.16 kcal mol^{-1} at Leu694 binding site, inhibitory constant of 0.036 µM and lipophilicity of 4.481. It was concluded from the data obtained from experimental results that A26 could bind with CTD of Hsp90α strongly and result *Tau* protein degradation which might be considered to be a therapeutic approach in AD.

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CHAPTER 1
INTRODUCTION

1.1 INTRODUCTION

1.1.1 Introduction to Alzheimer's Disease

Alzheimer's disease (AD) is a type of neurodegenerative disorder, its symptoms worsen as the time progresses thereby leading to irreversible intellectual and cognitive impairment, memory loss and ultimately to nerve cell death. With time, there are various ongoing researches which have given impeccable information about the disease, but so far there had been no cure for the disease. AD is not considered as the part of aging, when it occurs in individuals above 60, known as Late-onset whereas Early-onset occurs during the mid ages like 40s and 50s, but this is quiet uncommon among people [1]. In Earlier times, mentally ill people were considered to be insane or thought to be possessed by evil spirits. The treatments that were given to these people were very hard, but in the later period of time these people were taken care in the hospitals and the researchers began to determine the cause for the disease.

For understanding AD, it is important to have basic knowledge about the brain. There are number of cells present inside the brain, among them, neurons are highly electrically active cells which communicate with other nerve cells present within the brain through their dendrites and axons. When the dendrites of one neuron (pre-synaptic neuron) receive signals, they generate nerve impulse which travels along a neuron to the axon terminus and release a chemical known as neurotransmitter into a small gap known as synapse. From this gap the neurotransmitter is received by the receptors present at the boundary of post-synaptic neuron and as soon as it enters the interior of neuron it will work according to the received signal [2, 3].

AD mainly disturbs the chemical synapse scheme by disrupting the connection between the neurons leading to loss of metabolic activity and cell death. The death of neurons spread widely among nerve cells and the region of brain containing the dead neurons starts to shrink leading

to brain atrophy. Due to the death of neurons various symptoms can be observed in the patients such as mood swings, hallucinations, memory loss, orientation problem etc.

1.1.2 Causes of AD

The characteristic features of AD are the presence of two prominent unusual features in the brain which are made up of misfolded proteins – **amyloid plaques** and **neurofibrillary tangles** [4, 5]. These are abundantly present in the region of brain that is necessary for memory. Dr. Alois Alzheimer specified that amyloid plaques are formed outside the neurons due to the accumulation of amyloid beta-peptides. When normal brain ages, normal quantity of amyloid plaques deposit in the brain but in diseased condition the amount of these plaques increases than usual. It is unidentified that whether the disease is caused due to the accumulation of the amyloid plaques or these plaques are just the by-products of the disease processes.

1.1.3 Symptoms

It is believed that Alzheimer's disease develops in different ways but with many common symptoms in every individual and makes it very difficult to predict that how it will affect the person. One of the earliest symptom of the disease includes loss of memory but when the disease progresses, mood swings, aggression, confusion, long term memory loss etc. develops and the diseased person start becoming isolated from family and friends. Gradually, many body functions are gone which will ultimately leads to death. As AD can develop without being evident for uneven amount of time therefore it can progress undiagnosed for many years. The person suffering from Alzheimer's disease has the life expectancy of maximum seven years after getting diagnosed and very few persons can live more than fourteen year after diagnosis [6, 7].

1.1.4 Diagnosis

The initial diagnosis of Alzheimer's disease includes a series of tests and analysis to confirm the condition of the body including the brain. These tests include imaging techniques such as MRI, CT scan, PET scan which can detect neurological activity within the brain as well as the presence of any clump or an aberrant mass which may be indicative of amyloid plaques. The PET scan specially maps the regional activity of the brain through which we can confirm any abnormal loss of activity in the characteristic AD region such as the hippocampus. PET scan helps explorers to better diagnose the progression of Alzheimer's disease in different peripheries of the brain. Nowadays, Doctors and scientists are obtaining 3-D images of the brain by combining both PET (positron emission tomography) scan with MRI (Magnetic resonance imaging) to measure the rate different regions of the brain uses, deposits and metabolize certain chemicals. This has become possible with the advance of a radiolabeled compound called Pittsburgh Compound B (PiB). This PiB can bind to beta-amyloid plaques in the brain and it can be imaged using PET scans. A series of blood tests are also carried out to detect anomalous proteins in the vascular system. These tests are also followed by various cycles of psychological counselling of both the patient and kin to confirm any behavioural changes over the past period of time [8, 9, 10].

1.1.5 Current treatments of AD

Alzheimer's disease is regarded as an incurable condition, regardless of which many of the drugs are available in the market today which are prescribed associated with this disease, treat the symptoms rather than the disease itself. Lately, there are drugs that are being used to treat various symptoms of AD such as cholinesterase inhibitors, NMDA receptor antagonists, Antihypertensives, Antidepressants, Selective Serotonin Reuptake Inhibitors, Monoamine Oxidase (MAO) Inhibitors and anti-diabetes drugs.

Cholinesterase inhibitors such as tacrine, rivastigmine, galantamine, donepezil etc block the active sites of AChesterase thereby preventing the degradation of acetylcholine and results in efficient signal transduction [11]. These drugs have high toxicity and acute side-effects such as hallucinations, confusion, fatigue, dizziness, nausea etc. Hence, it is a requirement of new researches to come up with a drug with least side effects and one which treats the disease as a whole rather than just the symptoms [12].

NMDA receptor antagonist like memantine has been approved for the treatment of moderate to severe AD as the clinical trials showed improvements with and without cholinesterase inhibitor [13, 14].

Antihypertensives like β-blockers and Hydrochlorothiazide are observed to decrease the progression of AD. Unluckily, the studies which are related to hydrochlorothiazide are all concerned with the reduction of blood pressure upon progression of dementia rather than to detect anti-dementia MOA [15].

Antidepressant such as Amitriptyline is used to treat depression, migraine and neuropathic pain. In studies, it was observed from different cellular models that this drug binds to TrkA and TrkB which can prevent apoptosis or death of nerve calls (brain cells). Although, in this treatment with the decrease in toxic Aβ dimer there is an increase of non-toxic Aβ monomer thereby increasing the total Aβ in animal models [16].

Selective Serotonin Reuptake Inhibitors such as fluoxetine, Paroxetine and desvenlafaxine showed a decrease in levels of Aβ plaques in patients who had been taking these drugs [17]. Paroxetine is a drug that had been known to increase brain derived neurotrophic growth factor and reduce the levels of Aβ and p-*Tau* (phosphorylated *Tau*) thereby conserving the cognitive function in AD mouse models [18].

Monoamine Oxidase (MAO) Inhibitors like Selegeline and rasagiline are known for their neuroprotective property in both cellular and animal models. The mode of action of these is

based on preventing apoptosis of nerve cells by stopping the activation of caspase 3. Rasagiline is advantageous over selegeline as it does not have toxic metabolism like selegeline [19, 20]. Anti-diabetes drug like Metformin is used to treat type II diabetes mellitus when went through an experimental observations in AD models have produced mixed results. Upon administration of metformin alone, the generation of Aβ found to be increased by upregulating β-secretase 1. However, these levels were found to be reduced when the drug was administered along with Insulin. When metformin was administered in an insulin-resistant neuron model, it prevented neuropathological and molecular features of AD [21, 22].

1.1.6 Role of Hsp90α in the treatment of AD

Heat shock protein 90 plays a significant role in folding, stabilizing, activating and maintaining the conformational integrity of its client proteins like dcd4, B-Raf, Her2 etc through its ATPase activity. Hsp90α is a homodimer where each monomer consists of three different conserved domains namely a 25kDa N-Terminal domain (NTD) which contains a nucleotide binding site, 35kDa Middle domain (MD) which has client protein binding site and 12kDa C-Terminal domain (CTD) having a ATP binding and dimerization site (fig 1). Treatment of AD through drugs interacting with molecular chaperones focuses on the inhibition of Hsp90α thereby initiating the cascade of the degradation pathway rather than refolding of tangled HP-*Tau* proteins. One such mechanism to carry out this process includes the blockage of ATP binding sites at the CTD of the molecular chaperone and hence leads to the retardation of disease progression. As it is a well-known fact that *Tau* pathology is closely associated with the neuronal loss due to its ability to cause neurotoxicity. Therefore, Targeting *Tau* tangles would be advantageous to be used as a therapeutic strategy for treating AD.

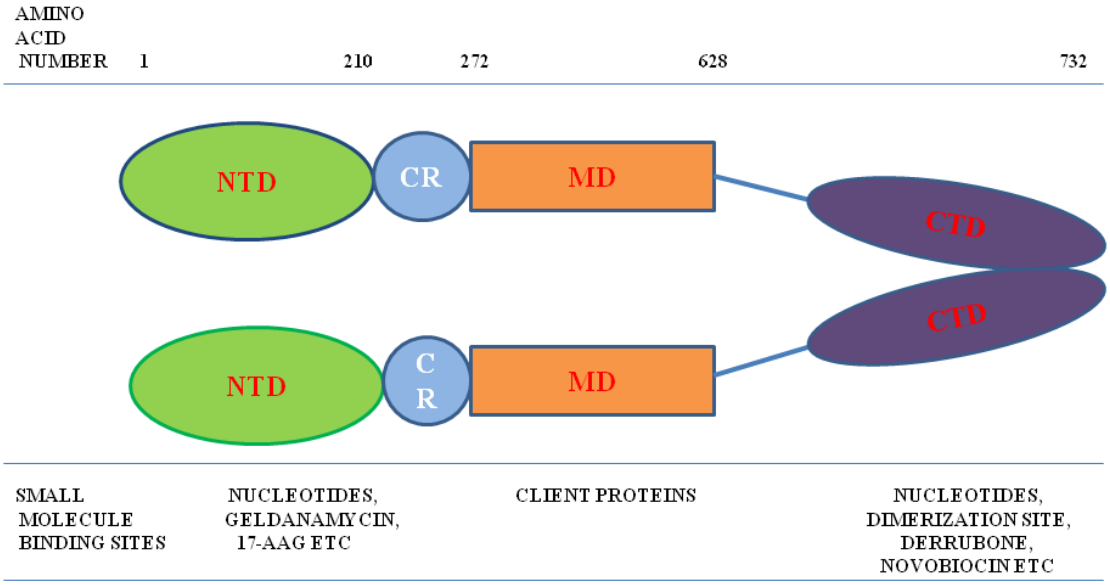

Figure 1: Structure of Hsp90α.

The use of these inhibitors for blocking Hsp90α could be a novel approach for the treatment of brain maladies like AD. Till date, there have been a number of experiments carried out which describes the role of Hsp90α inhibition for the treatment of AD [23, 24, 25]. Various inhibitors such as Geldanamycin (GD), 17AAG, 17DMAG etc of Hsp90α NTD have been identified but they have few drawbacks associated with them for which they could be used for the treatment of AD [26, 27, 28]. Due to the problems associated with the NTD inhibitors, various CTD inhibitors came into existence such as Novobiocin, clorobiocin, Derrubone etc [29-33]. Derrubone is a prenylated flavonoid which was isolated from a tree named Derris robusta [34]. Various In-vitro studies has determined Derrubone as a CTD inhibitor of Hsp90α which exhibits a novel mechanism of its inhibition by obstructing the ATPase activity of NTD and altering the client protein binding to MD through conformational changes in the protein structure. Furthermore, it was predicted that Derrubone could strongly bind to the CTD of Hsp90α and lead to *Tau* protein degradation which can be a therapeutic approach for the treatment of AD.

1.1.7 Bioinformatics and Computational Biology

Bioinformatics tools help us in a large way to examine and evaluate various biological processes which are otherwise hard to evaluate *in-vitro* or *in-situ* such as drug designing, evolutionary computation, numerical programing of enzymatic processes etc. Bioinformatics is an interdisciplinary branch of science that deals with drug designing processes. It has the potential to accelerate the process of drug discovery thereby reducing the cost of the process and giving different methods of designing a novel drug. Molecular docking is the most commonly used method for the identification and designing of novel drugs. It involves docking of the drug molecule to its biological target and the binding site for the drug is its site of action which gives the pharmaceutical effect of the drug. In molecular docking, the protein and the ligand are allowed to bind in a 3D space and give the binding energies and the inhibition constant value of the drugs. There are various software which can be used for molecular docking experiments after the retrieval of protein structure and drug molecule from online databases. The advancement in computational biology approaches has led to the development in drug discovery and helps the pharmaceutical sector progress towards better medical facilities and provide better health care.

1.2 OBJECTIVES

- Retreival of 3-D structure of C-Terminal domain of Hsp90α.
- Identification of possible ATP-binding sites of Hsp90α in the CTD using various methods and softwares.
- Calculation of binding affinities of various Hsp90-CTD inhibitors by performing molecular docking study at the determined ATP binding sites using AutoDock 4.0 software
- Development of novel analogue of Derrubone which can bind to the previously identified ATP binding sites of Hsp90α CTD thereby modifying its binding affinity, lipophilicity, water solubility and minimizing its Inhibition constant.
- The ADME and toxicity studies using Discovery Studio 4.0 software.
- Molecular docking of the analogues against Hsp90α CTD using Autodock 4.0 and the comparison of binding energies and inhibition constant to obtain a novel inhibitor of CTD Hsp90α.

CHAPTER 2
LITERATURE REVIEW

REVIEW OF LITERATURE

2.1 Alzheimer's Disease

Dr. Alois Alzheimer, who was a German neurologist, discovered Alzheimer's disease in 1906 after examining the brain of 51 year old patient who suffered from depression, dementia, paranoia, hallucinations etc, with peculiar formations in the brain (amyloid plaques and neurofibrillary tangles). It is believed that an urgent need of strategies to treat AD is required as it is highly spreading and largely devastating [5].

The starting material for the formation of amyloid plaques is the Amyloid Precursor Proteins (APP) which is tethered to the cell membrane. There are enzymes present in cellular compartments like alpha-secretase, beta-secretase, and gamma-secretase which cleave APP into discrete fragments. The cleavage of APP can proceed in two different types of pathways which can have two different consequences for the neuron i.e. favourable pathway and unfavourable pathway. In the harmful pathway, oligomers are formed due to the sticking of three, four or more beta amyloid plaques, resulting in enlargement of oligomers and formation of protofibrils and fibrils. Further, different proteins and other cellular materials also get attached to these insoluble entities and results in the occurrence of beta amyloid plaques [35, 36].

Another characteristic of AD is the formation of neurofibrillary tangles. The transport of nutrients and vesicles containing the neurotransmitters by healthy neurons is done through microtubules. These microtubules are stabilized by *Tau* proteins which have few phosphate molecules attached to them. GSK-3β is the most important enzyme which plays its role in phosphorylating *Tau* proteins either in normal or diseased condition. In AD the major component of tangles are *Tau* proteins and these proteins become three to four fold more phosphorylated than in normal brain of an individual. The HP-*Tau* leads to the reduction in

binding ability of MTs leading to its disorganization, subsequently *Tau* proteins form aggregates in the form of NFTs within the cytoplasm, which cause damages in the neuronal transport pathway. In the diseased condition, large number of phosphate molecules gets attached to the *Tau* protein and this hyperphosphorylation disengages the *Tau* from the microtubules. These *Tau* proteins cling with other *Tau* threads resulting in the formation of tangles inside the nerve cell. This causes the disintegration of microtubules and damages the transport pathway of the neurons by disrupting the communication between the neurons [37, 38].

AD is due to mutations that occur in APP (amyloid precursor proteins) and PS1 (presenilin). The main cause for this is polymorphism of APOE (apolipoprotein) gene. APOE is produced by microglial and astrocytes in brain cells and they have their role in clearance of amyloid present extracellular. Thus if the mutations occurs in APOE it reduces their efficiency to clear the Aβ peptide and result in accumulation of these leading to the progression of the disease. Later, it was revealed that upon reducing the amount of amyloid does not stop or halt the disease. Another approach of using a drug 'FLURIZAN' (ϒ secretase modulator) which inhibits PS1 was also remain ineffective in preventing the Aβ production. Hence, alternate stratergy was required which targeted on another cause of the disease that is *Tau* protein aggregation into tangles. Microtubule associated protein *Tau* (MAPT) gene was found to cause disease. It was found that *Tau* is capable of causing neurotoxicity therefore neuron loss was considered to be closely related to tangle pathology. Finally, Targeting *Tau* tangles was considered to be more effective strategy than anti- Aβ therapies [39].

2.2 Hsp90α and AD

The formation of HP-*Tau* can either be directed towards refolding, de-phosphorylation or proteasomal degradation when all other mechanisms fail [fig. 2]. In diseased condition, it is

supposed that these mechanisms become inactive and results in NFTs [40, 41]. HP-*Tau* not only has distorted conformation but it also has increased affinity for molecular chaperones like Hsp90α. Hence, *Tau* clearance can be achieved by developing the strategies which can target Hsp90α and related co-chaperones [42].

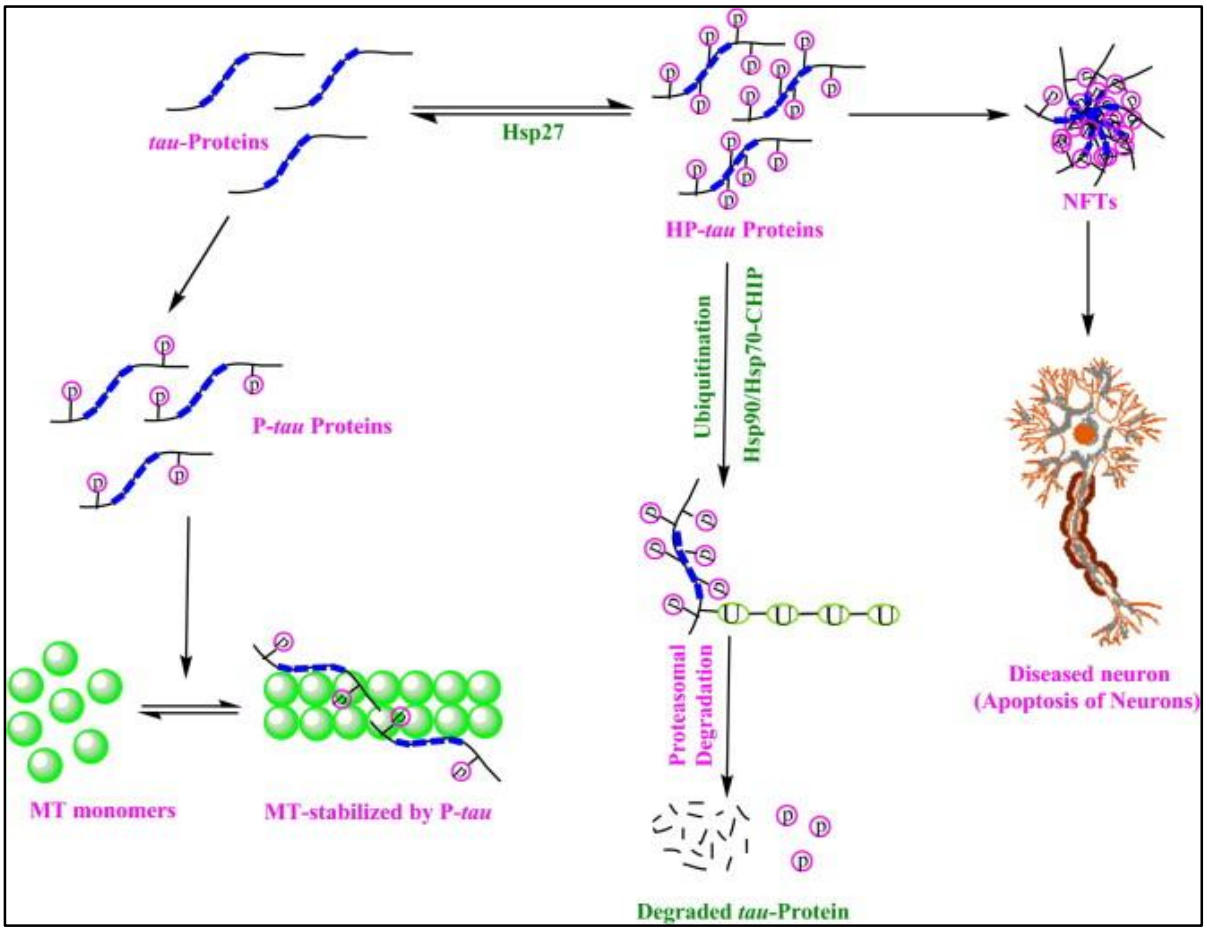

Figure 2: Pathway describing the stabilization of neuronal microtubules with the help of *Tau* protein. The activation of *Tau* proteins occur by their phosphorylation and their hyperphosphorylation can either lead to its dephosphorylation assisted by Hsp27, trigger to proteasomal degradation by Hsp90/70-CHIP complex or their deposition occurs as NFTs leading to neuronal atrophies [Khalid S and Paul S. Medical hypotheses 2014].

Hsp90α is a molecular chaperone which is inducibly expressed during the stress conditions like nutrient deprivation, malignancy, exposure to toxins etc. and is involved in the refolding of unfolded proteins with the help of co-chaperones like p23, PP5 etc. [43] or can otherwise

initiate the proteasomal degradation pathway. There are about 100 client proteins which have been identified as Hsp90-dependent client proteins [44]. The connection of Hsp90α with neurodegenerative diseases is basically due to its capability to refold aggregated proteins like HP-*Tau* proteins [45]. Therefore, it has been recognized that HP-*Tau* acts as a client protein for Hsp90α [46]. Drugs which bind to the ATP binding sites present in NTD and CTD of Hsp90α can inhibit the same thereby initiating the proteasomal degradation of HP-*Tau* in AD (fig. 3).

During the preliminary stage, when ATP is not bound to Hsp90α, NTD remains in an open conformation and the charged regions mask the CTD ATP-Binding site. Upon binding of ATP to NTD of Hsp90α, its conformational modifications occur and it exposes the ATP binding site of CTD. The binding of ATP to CTD follows with allosteric changes, causing the conformational readjustments rather than stimulating the ATPase activity [47, 48]. The binding of ATP to CTD leads to the dimerization of NTD along with the attachment of client proteins like *Tau* to MD which is assisted by Hsp70/Hsp40 complex. Subsequently, AHA1 and co-chaperones like p23, PP5 assists in hydrolysis of ATP and refolding of client protein, respectively and the NTD goes back to its open conformation state thereby releasing the folded *Tau* protein and ADP.

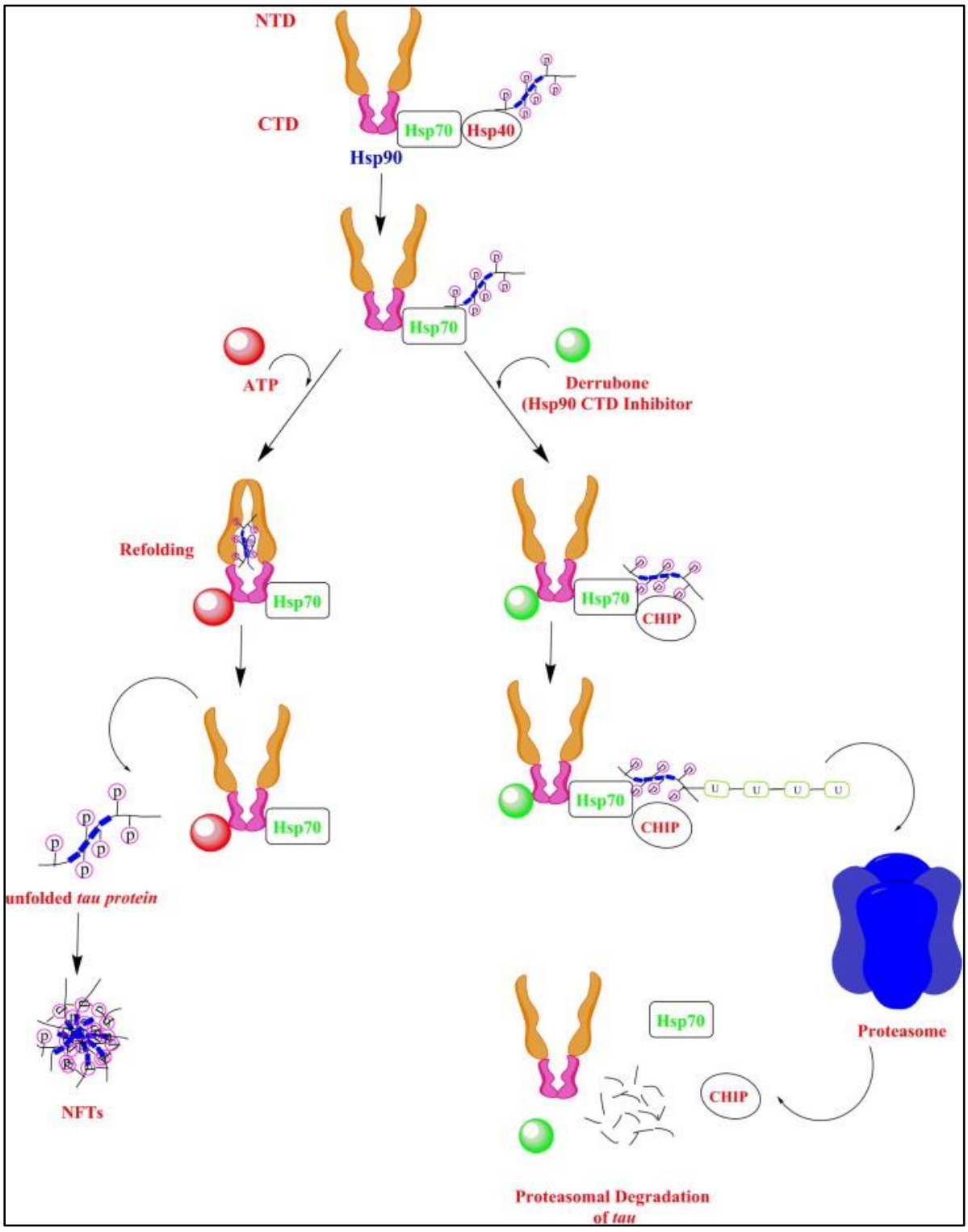

Figure 3: Mechanism of *Tau* tangles repair by Hsp90*α*. The molecular chaperone can subject the hyper-phosphorylated *Tau* tangles to refolding or proteasomal degradation depending on the attachment of ATP. ATP activation of Hsp90*α* renders the *Tau* protein to refolding process and the inactivation of the same leads to degradation of the protein through ubiquitination by CHIP protein [Khalid S and Paul S. Medical hypotheses 2014].

In the treatment of AD, when the inhibitors are allowed to bind to the ATP binding site of Hsp90α rather than ATP itself, the complex of HP-*Tau* protein with Hsp70/Hsp40 interacts with Hsp90α thereby allowing the recruitment of CHIP and directing the HP-*Tau* protein to proteasomal degradation [49, 50, 51]. There exist various drugs which can inhibit Hsp90α by blocking the ATP binding site of NTD, obstructing the client protein binding site of MD or by inhibiting CTD [52, 53]. The Hsp90α inhibitors bind to these domains and block its activity to stabilize or refold client proteins which are thereby degraded by Ubiquitin-proteasome pathway [54, 55]. The folding mechanism of client proteins by Hsp90α is carried out upon dimerization of CTD which is followed by the hydrolysis of ATP molecules. Hence, it is believed that the inhibition of CTD of Hsp90α might be a significant approach in various neurodegenerative diseases like Alzheimer's disease (AD) [40]. The binding of ATP has also been reported in the C-Terminal domain of Hsp90α [39].

2.3 Hsp90α Inhibitors

GD has very good binding affinity with Hsp90α but it shows poor water solubility and high hepatoxicity which later lead to the development of its soluble derivatives namely 17AAG (17-N-Allylamino-17-demethoxygeldanamycin) and 17DMAG (17-Dimethylaminoethylamino-17-demethoxygeldanamycin) [58]. There are various drawbacks associated with these derivatives such that 17AAG, despite being less toxic, has less binding affinity than GD for Hsp90α [26, 27] and 17DMAG has problems associated with drug clearance due to limited metabolism and it could not be used for prolonged period [59, 60].

To resolve the problems associated with NTD drugs, various CTD binding drugs such as Novobiocin, EGCG (Epigallocatechingallate), Derrubone, Clorobiocin etc. were also discovered later [29-33]. Novobiocin which was observed to bind CTD nucleotide binding site also showed very weak appearance of inhibition in cellular-based assays (IC50 ca. 500–700

μM) when compared to the inhibitors of NTD (IC50 < 100 nM) and it also has limited water solubility [61, 62]. As the folding mechanism of client proteins by Hsp90α is carried out upon dimerization of CTD which is followed by the hydrolysis of ATP molecules. Therefore, the inhibition of CTD of Hsp90α is being a significant approach in various neurodegenerative diseases like Alzheimer's disease (AD) [56]. The binding of ATP has also been reported in the C-Terminal domain of Hsp90α [57].

The crystal structure of Hsp90α CTD does not currently exist in the databases. The prokaryotic Hsp90α has been found to have 40-60% similarity with human Hsp90α (hHSP90α) [63, 64]. It has been known that the truncated CTD of Hsp90α could bind molecules with comparable affinity as that of fully intact Hsp90α [65].

2.4 Properties of a CNS Drug required to cross BBB

As AD is a brain malady, it is required that the drug should pass through the blood brain barrier (BBB), that is made up of tight junctions between the epithelial cells surrounding the αbrain tissue. The central nervous system (CNS) drugs should satisfy various properties of pharmacokinetic profile for crossing the BBB for which a set of rules are derived from about 1500 drugs, which includes

- lipophilicity (AlogP<5) : AlogP of a drug is the optimized octonal/water partition coefficient, which gives the measure of its lipophilicity that is an important factor for its oral absorption owing to its ability to penetrate through the cell membrane of the epithelia. Hydrophilic and lipophilic values of a drug which increase beyond a certain value are not considered ideal for CNS drugs. The drugs must be polar to be soluble in water, fatty to be able to cross BBB to avoid rapid excretion, weak bases are preferable and it must have lipophillic and hydrophilic character. AlogP of a drug is the optimized octonal/water partition coefficient, which gives the measure of its lipophilicity that is

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- Polar surface area (PSA<60-90 $Å^2$): Polar surface area denoted as $Å^2$, is the surface area that is occupied by oxygen and nitrogen atoms and the polar hydrogens that are attached to them. It gives the hydrogen bonding capacity and polarity of a drug. Many investigators has been used PSA as a predictor for BBB penetration [67, 68].
- Molecular weight (<450): this parameter is considered to be critical for BBB penetration. The molecular weight of CNS drugs is less than that of other therapeutic drugs.
- Number of rotatable bonds (<8): Rotatable bonds give the molecular flexibility of a drug and it is known that the CNS drugs have significantly fewer rotatable bonds as compared to the non-CNS drugs.
- Hydrogen bond donors (<3) and acceptors (<7): are responsible for the formation of hydrogen bonds between the drug and the protein molecule [66].

The optimization of the drug candidate with respect to its absorption, distribution, metabolism and excretion (ADME) is the most important part of drug designing thereby defining the therapeutic efficiency and character of a drug [67, 68, 69].

Absorption property for a particular compound is required when it not given to the patient directly into the bloodstream through intravenous administration. Hence, it is important for a drug that it should be transported to the circulation system from the site of administration, as it is considered to be absorbed when it is has entered the blood circulation. The absorption of

drugs solely depend upon two factors i.e. lipophilicity and solubility of a drug molecule. Factors like chemical instability, poor compound solubility, inability to permeate intestinal wall could lead to poor absorption.

Distribution property of a drug is required once it has entered the blood stream. The drug is allowed to get distributed across the body after absorption. This property of a drug could not be calculated directly therefore it is given by a relationship between the concentrations of drug present across the body to the amount of drug present in the blood for distribution at the same time. Factors affecting distribution are the poor blood flow rate, molecular size, polarity etc.

Metabolism of a drug is required for making the drug more soluble in water by breaking it into small metabolites, so that it could be excreted out easily from the body. The oxidation-reduction, hydrolysis and conjugation are the reactions which are involved in the metabolism of a drug candidate. The enzymes that are required for the metabolism of a drug are mostly located in the liver eg Cytochrome P40 enzyme for oxidative metabolism.

Excretion is another very important property required by a drug for their removal from the body, which can be achieved by kidney (urine), feces (bilary excretion) or lungs (anaesthetic gases). Other routes of elimination include the liver, sweat, and saliva [70].

The structure activity relationship (SAR) of Derrubone has led to the development of analogues which could give better binding affinity with lesser inhibition constant (ki) than DB [71] along with better lipophilicity and aqueous solubility. The solubility of the drug could be increased by addition of polar groups like alchohol, amide, amine, carboxylic acids etc as they are capable of making strong intermolecular forces of attraction like hydrogen bonds. The lipophilicity of a drug can be enhanced by increasing the length of the alkyl chain attached to the basic structure of the drug.

CHAPTER 3

MATERIAL AND METHODS

3.1 MATERIALS

3.1.1 Tools and bioinformatics softwares used:

- Protein Data Bank (www.rcsb.org/pdb/home/home.do)
- Pubchem (pubchem.ncbi.nlm.nih.gov)
- PRODRG Server (davapc1.bioch.dundee.ac.uk/cgi-bin/prodrg)
- PHYRE 2 Server (Protein Homology/analogY Recognition Engine)
- ArgusLabs
- Chimera 1.6.1
- Open babel GUI software
- ATPint
- Ligplot+ v1.4.5
- Protein Hydrophobicity Plot (www.vivo.colostate.edu/molkit/hydropathy/)
- Autodock 4.0
- Hex 6.3
- ACD/ChemSketch
- ChemBioDraw Ultra 13.0
- MedChem Designer
- Discovery studio 4.0

3.2 METHOLOGY

3.2.1 Retrieval of 3D structure of C-terminal Hsp90α and drugs

The present experiments have been carried out by utilizing the 3-D structures that are retrieved from PHYRE2 server. The server prepared the structure from homology modelling of prokaryotic Hsp90α which is believed to be 40–60% similar to hHsp90α (human). Following are the steps involved in the retrieval of the 3D structure of Hsp90α CTD:

The URL www.sbg.bio.ic.ac.uk/phyre2/ was opened for PHYRE2 Server.

↓

The E-Mail address and job description is given in the space provided.

↓

The residues from 628-732 are pasted in the amino acid sequence box followed by the running of normal PHYRE2 Search.

↓

The result obtained from the PHYRE 2 server is sent to the provided E-Mail address

The 3D structures of drugs or Hsp90-inhibitors were retrieved from PubChem followed by their energy minimization in PRODRG server. The steps involved in obtaining the structures of the inhibitors are mentioned below:

After opening the URL pubchem.ncbi.nlm.nih.gov/, the compound name was entered in the space provided.

↓

For obtaining the 3D conformation of the drug, 3D SDF display was opened and copied.

↓

PRODRG URL davapc1.bioch.dundee.ac.uk/cgi-in/prodrg/ was opened and the 3D

↓

SDF display coordinates obtained from PubChem were pasted in it.

↓

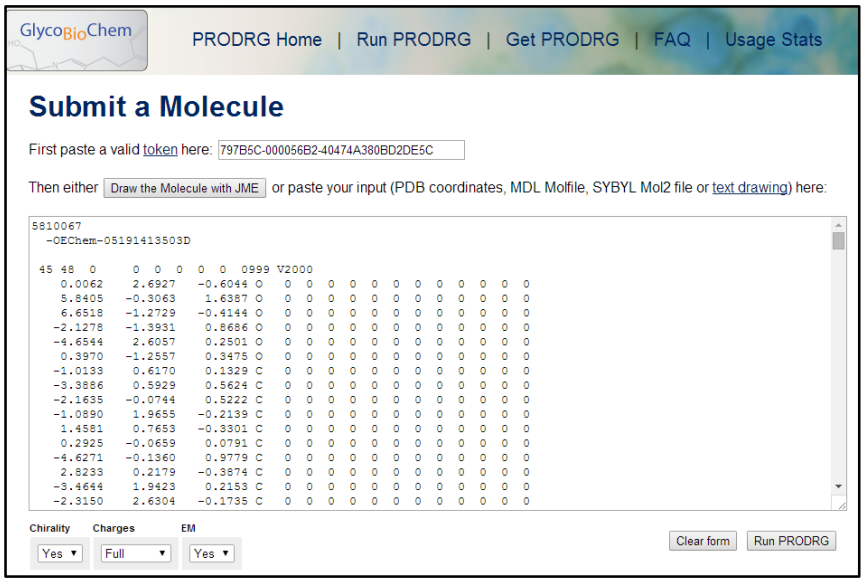
With charges keeping full, PRODRG was run.

↓

Finally, only the PDB file (polar hydrogens) box was selected from the output and saved in .pdb file format in word pad.

↓

3D structures of drugs such Novobiocin, Clorobiocin, Derrubone, EGCG etc were obtained from PubChem server.



3.2.2 Identification of ATP binding sites in C-Terminal domain (CTD) of Hsp90α using various software

3.2.2.1 ATPint

it is an online Server used for predicting the ATP binding sites from aprotein sequence of a molecule. The server works with the help of Support Vector Machine (SVM) and Position-specific scoring matrices (PSSM) generated by psi-BLAST. The following are the steps involved in the prediction:

URL www.imtech.res.in/raghava/atpint/submit.html was opened

↓

The sequence name along with the E-Mail address was entered

↓

Amino acid sequence from 628-732 amino acids of Hsp90α CTD was input in FASTA format

↓

The software was run and the results were sent to the E-Mail ID provided.

3.2.2.2 CAST P calculation

It is also an online server that is used to predict the binding sites of a protein molecule. According to this software the possibility of ATP binding sites is expected to be present in the largest cavity or pocket of the input 3-D structure of Hsp90α. The steps involved in determining the active sites are Firstly, sts-fw.bioengr.uic.edu/castp/calculation.php browser was opened and the protein structure was input in .pdb file format.

3.2.2.3 Hex 6.3

Hex 6.3 is molecular docking software which gives the value of binding energy upon interaction of receptor and ligand molecules. The binding energies results obtained from the software is calculated upon addition of all the intermolecular forces that could be possibly

occurring between the receptor and the drug molecule. Rigid as well as flexible docking could be performed using the software. In the process of obtaining the binding energies, a grid box is created on a named atom of protein molecule i.e the recognized ATP binding site, on which the drug molecule interact followed by docking. In the software, the 3-D structure Hsp90α CTD and various drugs were input in the .pdb format. Docking option was selected from the control tab and run.

3.2.2.4 LigPlot+ v.1.4.5

It is used to obtain the 2D images of the docked complexes by providing the interacting residues and bonds that are present in the vicinity of the binding site of ligand and protein molecule. The 3D structure of protein-ligand docked molecule (Hsp90α CTD-drug) is input in the software in .pdb file format. In the LigPlot analysis, the interpretation of the result obtained was done by using the following information.

- The residues which were denoted by red coloured arc showed the hydrophobic residues of the protein molecule that interacted with the drug molecule.
- The oxygen, carbon and nitrogen atoms were represented by red, black and blue color spheres, respectively, of the drug molecule whereas the purple colored lines represented the bonds that were present within the drug molecule.
- Blue colour bonds presented between the drug and the protein molecule were the external bonds.
- Bonds which were coloured green indicated the hydrogen bonds that were formed between the drug and protein.

3.2.2.5 Protein hydrophobicity plots

The software predicts the hydrophobic character of input protein sequence. This program utilizes use of two types of scales; the Kyte-Doolittle scale which indicates the hydrophobic amino acids, and the Hopp-Woods scale that gives the hydrophilic residues. The current work utilizes the Kyte-Doolittle scale with window size set to 19, which displays the most hydrophobic residues in the Hsp90α CTD input sequence.

3.2.3 Molecular docking by AutoDock 4.0 software

Molecular docking experiments were carried out using Autodock 4.0 which provided the binding affinity of Hsp90α along with the inhibition constant with various analogues. The software is based on Lamarckian Genetic Algorithm (LGA) giving the lowest energy of the system for becoming stable. In the present work, the Autodock 4.0 is used for docking of various CTD drugs and newly designed analogues of Derrubone against Hsp90α CTD to find out the best binding inhibitor with smallest value of inhibition constant.

The Hsp90α CTD and the energy minimized drug were docked using the software AUTODOCK 4.0 through the following steps:

a) **Protein target** :

   i. File -> Read molecule -> Hsp90α

   ii. Edit -> Hydrogens -> Add Polar only

   iii. Edit -> charges -> Add kollmann charges

   iv. Edit -> atoms -> Assign AD4 type

   v. File -> saved in Hsp90α.pdbqt format

b) **Ligand file** :

i. Ligand -> Input Molecule -> Read Molecule

ii. Ligand -> torsion tree -> choose root (manual selection of root atom)

iii. Ligand -> torsion tree -> detect root (automatic detection of the root that provides the smallest largest subtree).

iv. Ligand -> output -> save as drug-name.pdbqt format

c) **To prepare macromolecule file** :

i. Grid -> Macromolecule -> Macromolecule target (Hsp90α)

ii. Grid -> Set Map Types -> Choose Ligand -> selected Ligand (drugs)

iii. Grid -> Grid Box (in the **Grid Options Widget** the active site for ATP binding was selected to centre the grid box on the active site and the number of points in each dimension were adjusted to 60).

iv. Close saving current.

v. Grid -> output -> save as Hsp90α.gpf

d) Autogrid

i. Run -> Run AutoGrid. The Run AutoGrid widget opened in which **Program Pathname** (the location of the autogrid3 executable) and **Parameter Filename** (Hsp90α.gpf file) was specified. **Log Filename** will be specified in Hsp90α.glg format automatically (selecting a gpf creates a possible related name for the glg).

ii. Launch

e) **Prepare docking parameters** :

i. Docking -> Macromolecule -> Rigid Filename (Hsp90α)

ii. Docking -> Set Ligand Parameters -> Choose Ligand (drug)

iii. Docking -> Set Search Parameters -> Genetic Algorithm Parameters

iv. Docking -> output -> Lamarckian Genetic Algorithm (LGA). Save in Hsp90α.dpf.

f) **AUTODOCK** :

i. Run -> Run AutoDock. This Opened the Run AutoDock widget in which **program Pathname** (the location of the autodock4 executable) and **Parameter Filename** was specified (Hsp90α.dpf file format). **Log Filename** will be specified automatically in Hsp90α.dlg format (Selecting a dpf creates a possible related name for the dlg).

ii. Launch

g) **Obtaining rmsd value and inhibitor constant**: open the Hsp90α.dlg file in word pad and copy the atoms corresponding to the highest binding energy and paste it in the end of cleaned protein (Hsp90α) text file. Save the word file in unicode text document as Hsp90α.pdb. Further the Hsp90α.pdb was opened in Chimera software for visualization of the hydrogen bonds.

h) **Visualization of hydrogen bonds** :

Open -> chimera

Select -> residue -> drug

Action -> focus

Tools -> stucture analysis -> find H-bonds

This will give all the number of hydrogen bonds formed between the drug and the protein (Hsp90α) along with the bond length. Residues forming these bonds can also be known through this software.

The picture is then copied using the print screen option and paste in paint to crop and save the image in .jpeg or .png format.

3.2.4 Design of Derrubone analogues

The designing of the analogues of Derrubone was carried out using ACD/Labs ChemSketch software (ACD/Structure Elucidator, version 12.01, Advanced Chemistry Development, Inc., Toronto, ON, Canada, www.acdlabs.com, 2014). The designing of analogues was carried out taking into account the properties such as lipophilicity and solubility. The solubility of the analogues was improved by adding functional groups like R–OH, R-COOH, R-COO-R', R-CO, R-NH_2 etc. whereas the lipophilicity was enhanced by groups such as R-O-R' and by increasing the length of alkyl chain.

3.2.5 ADME and Toxicity Studies of Derrubone Analogues

The computer aided ADME studies and toxicity studies have been done by using the software Accelrys Discovery Studio 4.0 Software. The molecular structure of the query compound (Analogues prepared) is input in Mol file format. The investigation is solely based on the chemical structure of a compound assisted in determining the BBB penetration, polar surface area, aqueous solubility, human intestinal absorption (HIA) etc for a specified set of drug candidates. ADME-Plot is a 2D graph plotted against Alogp98 vs. PSA. The graph consists of ellipses which are derived from about 800 drugs that can enter CNS after their oral

administration and they show the regions where the compound is well absorbed [Egan and Lauri, 2002]. There is no prediction made for the compounds that fall outside the 95% and 99% confidence ellipses. From the studies, it is known that the candidate will fall within the 95% ellipse region if it is found to be 95% of the well absorbed compounds whereas the 99% of the well absorbed compounds will fall in the 99% ellipse. This PSA_2D vs AlogP98 graph predicts the penetration of BBB as well as the 95% and 99% confidence ellipses of the compound after the oral administration. In BBB, the ellipses are not similar to those of human intestinal absorption but they have equivalent interpretation. TOPKAT (Toxicity Prediction Komputer Assisted Technology) study determines toxicity based on the 2-D structure of a compound. It evaluates the Rodent Carcinogenicity, Mutagenicity (Ames test), Aerobic Biodegradability and Skin irritation. In the current study, the ADME and toxicity profiles of Derrubone and its analogues were carried out to determine a novel inhibitor which can inhibit Hsp90α and could be used as a therapeutic drug for treating AD.

CHAPTER 4

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

4.1 Identification of ATP binding sites on C-Terminal domain of Hsp90α

The ATP binding site of the CTD of Hsp90α is not known; hence we predicted the possible ATP binding sites present in this domain using various online servers and. From ATPint software, we obtained Leucine(Leu) 694 as an ATP interacting residue of the CTD of Hsp90α. The validation of the result obtained from from ATPint, CastP calculation was done. From the result of CastP, it was observed that Leu 694 and Leu 665 were present in the largest binding pocket of Hsp90α CTD (fig. 4).

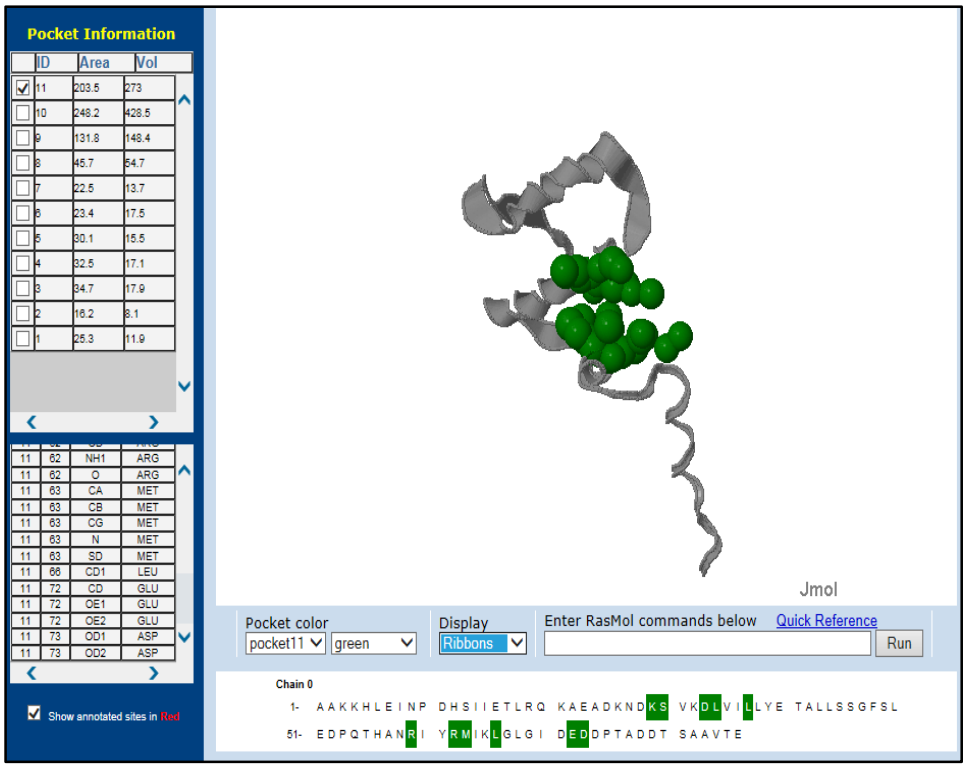


Figure 4: CastP Calculation showing the presence of Leucine 694 (66) and Leucine 665 (37) in the largest binding pocket of Hsp90α.

Furthermore, for validation Hex 6.3 was used for performing molecular docking experiments of novobiocin and ATP molecule against CTD Hsp90α. The docked results obtained from the software were analysed using LigPlot+ 1.4.5. Software (fig. 5a and 5b). The residues denoted

by red coloured arc are the hydrophobic residues of Hsp90α that are interacting with the ATP and drug molecule, respectively. Red, blue and black colour spheres indicate the oxygen, nitrogen and carbon atoms, respectively, of the ligand molecule whereas the bonds are represented by purple coloured present within the drug candidate. External bonds between the drug and Hsp90α are represented by blue colour. The hydrogen Bonds are represented by green colour that are formed between the drug and Hsp90α. From Ligplot analysis, it was known that both ATP and Novobiocin were found to be interacting with Leu694 which was previously validated by ATPint and CastP calculation. The bonds formed and the interacting residues of Hsp90α CTD with Novobiocin and ATP were observed to be identical. These results further validated the hypothesis that Leu 694 could be the binding residue for ATP along with two other hydrophobic residues Leu 665 and Leu 666. As it is a well-known fact, that the ATP and various other ATP binding site-inhibitors bind to hydrophobic and non-polar residues in the region [72].

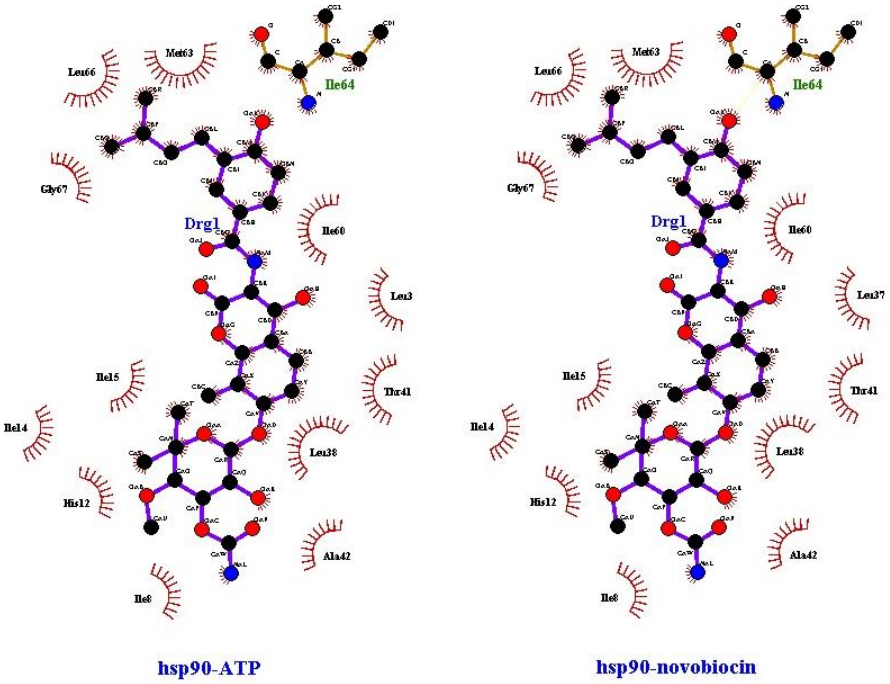


Figure 5a. Ligplot analysis showing the interaction of Hsp90α CTD residues with different ligands (i) ATP and (ii) novobiocin.

Furthermore, when Hydrophobicity plot was acquired from the software, it was observed that residues from 656 to 668 in CTD of Hsp90α falls under the hydrophobic region of the plot and further, it was observed that our previously determined sites Leu665 and Leu 666 comes in that hydrophobic region (fig. 6)

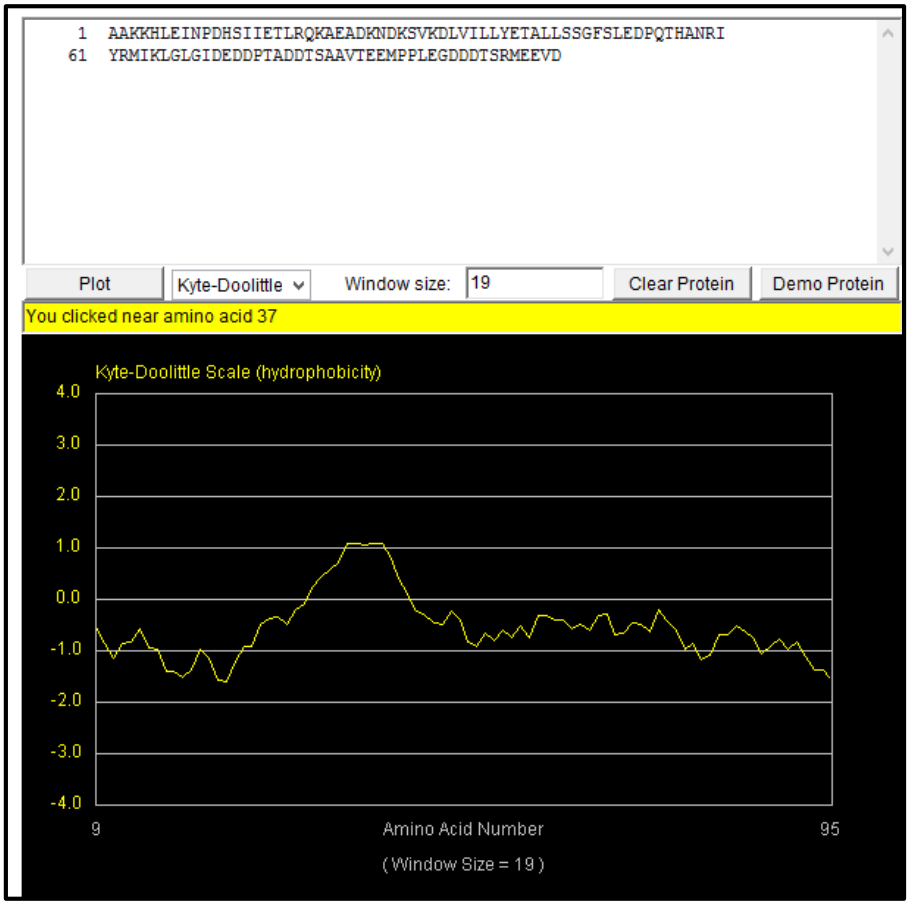


Figure 6: Hydropathicity plots showing the hydrophobic regions of Hsp90α CTD.

4.2 Docking of Hsp90α CTD with its existing inhibitors

Autodock 4.0 software was used for molecular docking experiments in order to obtain the binding affinities of various drugs and their inhibition constants against Hsp90α. The experiment was conducted by docking various existing CTD inhibitors on the previously recognized ATP interacting sites i.e Leu 665, Leu 666 and Leu 694, respectively. The binding energies and the Inhibition constants values obtained from the above experiment are tabulated

in Table 1. The results generated from docking experiments clearly showed that Derrubone demonstrates lowest binding energy and the inhibition constant for all the three sites of ATP interaction; followed by Novobiocin, Clorobiocin and EGCG. Further, Derrubone had the best binding energy and inhibition constant value at Leu 666 (-7.53 kcal/mol and 3.04μm) in comparison to Leu 665 (-7.20 kcal/mol and 5.31μm) and Leu 694 (-6.67 kcal/mol and 12.96μm).

Table 1: Docking of Hsp90α CTD with ATP and various inhibitors at three different ATP binding sites on Hsp90α CTD.

LIGAND	RECEPTOR	LEUCINE 665		LEUCINE 666		LEUCINE 694	
Drug		Binding energy (kcal/mol)	Inhibition constant (k_i) (μm)	Binding energy (kcal/mol)	Inhibition constant (k_i) (μm)	Binding energy (kcal/mol)	Inhibition constant (k_i) (μm)
ATP	Hsp90α C-Terminal domain	-1.68	-	-2.34	-	-3.51	-
Derrubone		-7.20	5.31	-7.53	2.40	-6.67	12.96
Novobiocin		-5.03	205.3	-7.00	7.34	-5.79	52.76
Clorobiocin		-6.59	14.72	-6.32	23.28	-6.15	31.15
EGCG		-5.52	90.20	-5.65	72.16	-5.13	174.35

4.1.3 Docking of Hsp90α CTD with Derrubone

As it was analyzed from previous experiments that Leu 665, Leu 666 and Leu 694 could be best binding sites of Hsp90α for Derrubone. Further, the docking experiment was performed using Hex 6.3 between Derrubone and Hsp90α to validate the results obtained from previous experiments. This experiment was followed by LigPlot+ v.1.4.5 analysis to validate that whether our drug interacts with these sites or elsewhere other than the site of interest. From the

LigPlot+ v.1.4.5 analysis, it was observed that Derrubone (with the best binding energy) also interacts with Leu 665 and Leu 666 residues of CTD (fig. 7) similar to ATP and Novobiocin.

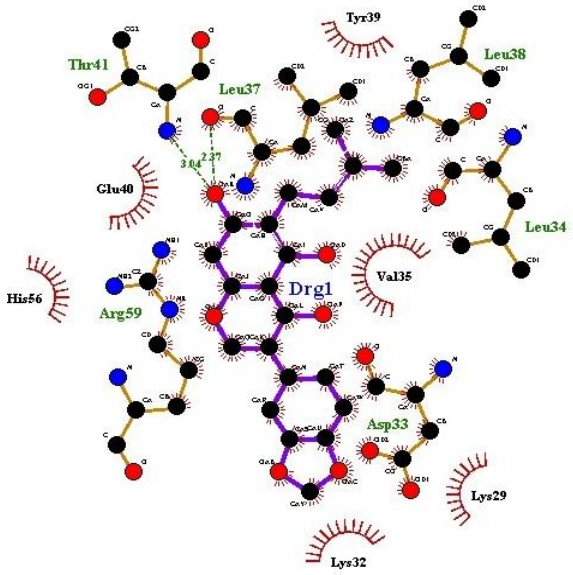


Figure 7. Hsp90α CTD and Derrubone interacting residues.

4.4 ADME and Toxicity studies of existing drugs and newly developed Analogues using Discovery Studio 4.0.

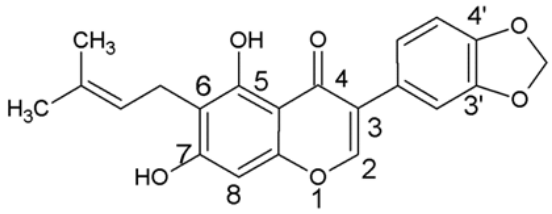
In the present work, the ADME and toxicity profiles of various NTD, CTD blocking drugs including Derrubone and its 30 designed Analogues were performed using Discovery studio 4.0 to determine a potent inhibitor which has the recommended properties as required for crossing BBB and treating brain maladies like AD. For comparative studies, various properties such as molecular weight, lipophilicity (AlogP), polar surface area (PSA), rotatable bonds, hydrogen bond donors and acceptors were calculated for each analogue and the existing drugs (Table 2). Results obtained from TOPKAT studies showed that among the existing drugs Derrubone has the best AlogP value while the others have either low or very high AlogP value as compared to the recommended value. It also has favourable PSA, number of rotatable bonds,

hydrogen bond donors and acceptors. Hence, it can be observed from this analysis that Derrubone is best for its use as a CNS drug. Additionally, when TOPKAT studies of Derrubone and its 30 analogues were carried out, it was observed that Analogue 1, Analogue 2, Analogue 3, Analogue 6, Analogue 7, Analogue 8, Analogue 9, Analogue 10 and Analogue 26 have improved lipophilicity than Derrubone, while keeping its Molecular weight, PSA, number of rotatable bonds, hydrogen bond donors and acceptors within the recommended range as required, for being a potent CNS drug [Table 2]. The structures of the above mentioned nine analogues are shown in (fig. 8) and the alterations made in the chemical structure of Derrubone to prepare these analogues have been encircled in the structure.

Table 2 ADME properties of 30 analogues prepared.

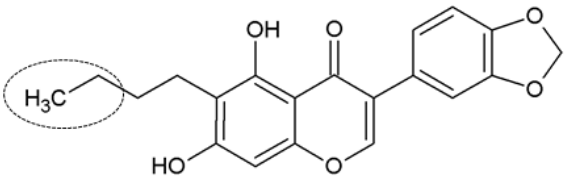
INHIBITOR	MOLECULAR WEIGHT (<450)	AlogP (<5)	Polar Surface Area (PSA) (<60-90 A^{o2})	ROTATABLE BONDS (<8)	HYDROGEN BOND ACCEPTORS (<7)	HYDROGEN BOND DONORS (<3)
Derrubone	366	4.007	85.122	3	6	2
Clorobiocin	697	5.034	185.22	10	11	5
Novobiocin	612	3.459	196.16	9	11	5
EGCG	458	3.096	197.37	4	11	6
Geldanamycin	560	2.364	163.43	5	9	3
17AAG	585	2.534	166.28	7	9	4
Analogue 1	**354**	**4.005**	**89.13**	**4**	**6**	**2**
Analogue 2	**288**	**4.119**	**70.67**	**4**	**4**	**2**
Analogue 3	**302**	**4.575**	**70.67**	**5**	**4**	**2**
Analogue 4	356	0.639	141.12	3	8	4
Analogue 5	370	2.041	115.37	4	8	1
Analogue 6	**380**	**4.233**	**78.13**	**4**	**6**	**1**
Analogue 7	**394**	**4.581**	**78.13**	**5**	**6**	**1**
Analogue 8	**380**	**4.233**	**78.13**	**4**	**6**	**1**
Analogue 9	**276**	**4.117**	**70.67**	**5**	**4**	**2**
Analogue 10	**408**	**4.807**	**67.13**	**6**	**6**	**0**
Analogue 11	274	3.663	70.67	3	4	2

Analogue 12	290	2.437	90.91	4	5	3
Analogue 13	303	2.597	82.70	5	5	3
Analogue 14	317	3.115	73.91	5	5	2
Analogue 15	318	2.611	96.97	5	6	2
Analogue 16	342	1.847	109.37	3	7	3
Analogue 17	356	1.815	126.32	3	8	3
Analogue 18	341	1.577	115.105	3	7	3
Analogue 19	355	2.009	101.23	4	7	3
Analogue 20	369	2.545	92,37	4	7	2
Analogue 21	354	1.915	106.20	3	7	2
Analogue 22	304	2.345	107.81	4	6	3
Analogue 23	289	2.147	96.87	4	5	3
Analogue 24	304	1.259	122.81	4	6	4
Analogue 25	302	2.485	87.71	4	5	2
Analogue 26	**394**	**4.481**	**78.13**	**5**	**6**	**1**
Analogue 27	264	-0.282	122.72	4	6	4
Analogue 28	277	2.146	96.96	5	5	3
Analogue 29	277	2.146	96.76	5	5	3
Analogue 30	410	3.901	87.36	6	7	1



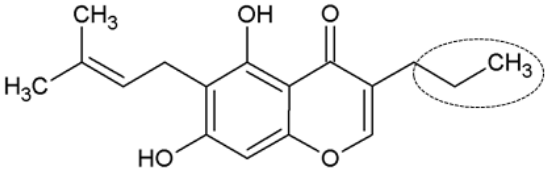
3-(1,3-benzodioxol-5-yl)-5,7-dihydroxy-6-(3-methylbut-2-en-1-yl)-4*H*-chromen-4-one

Derrubone



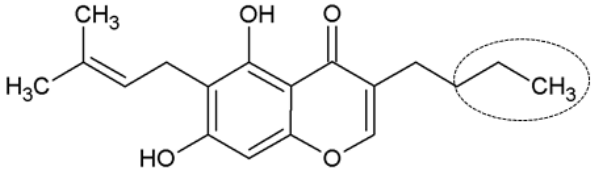
3-(1,3-benzodioxol-5-yl)-6-butyl-5,7-dihydroxy-4*H*-chromen-4-one

Analogue 1



5,7-dihydroxy-6-(3-methylbut-2-en-1-yl)-3-propyl-4*H*-chromen-4-one

Analogue 2



3-butyl-5,7-dihydroxy-6-(3-methylbut-2-en-1-yl)-4*H*-chromen-4-one

Analogue 3

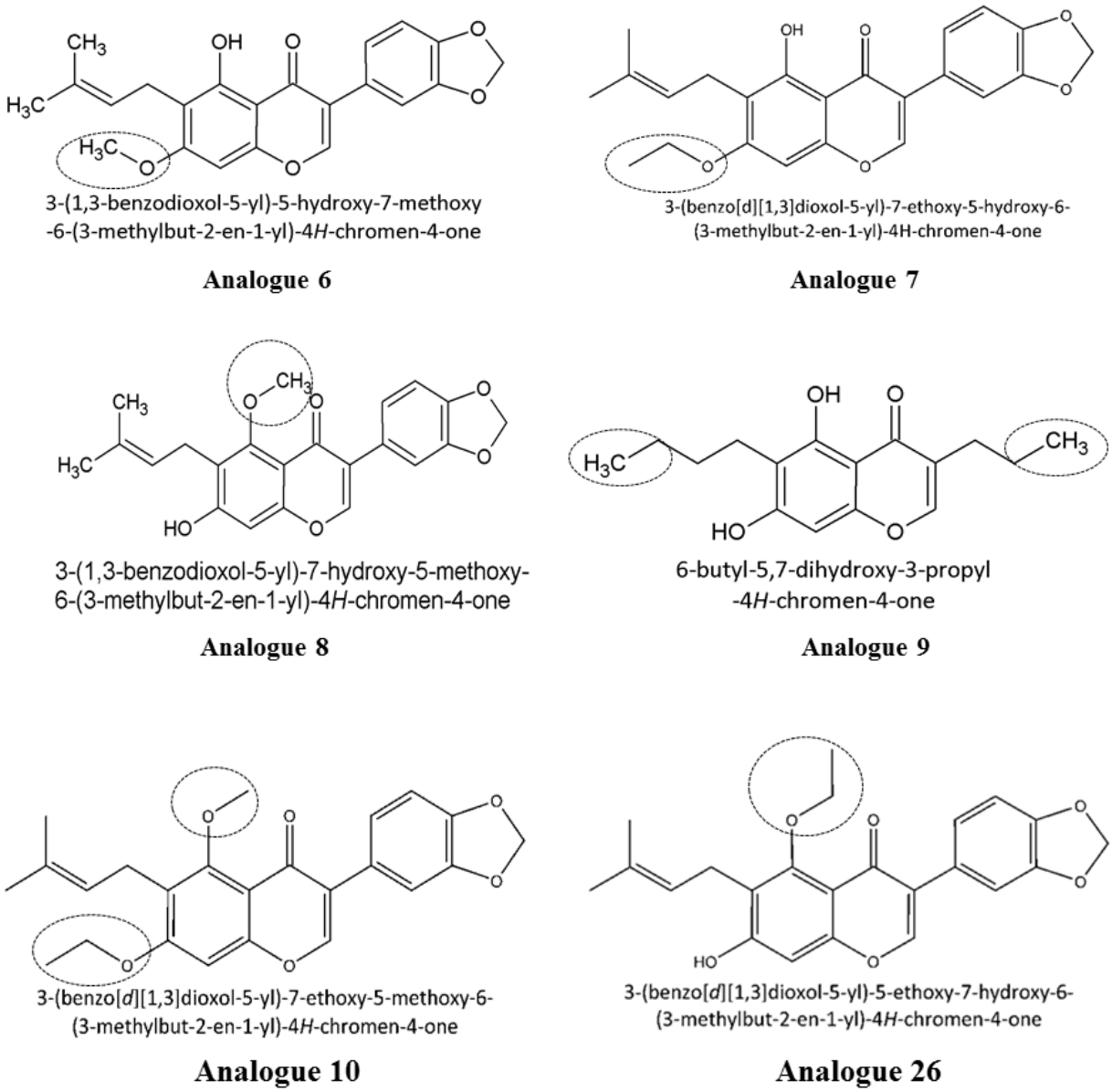


Figure 8. Structures of best nine analogues developed.

Among the newly designed analogues, nine of these were found to be better than Derrubone from the analysis of the results obtained from TOPKAT studies and were selected for further studies. Furthermore, the Extensible Toxicity Protocol was used to evaluate several other properties of the selected analogues including Derrubone, such as aerobic biodegradability, AMES mutagenicity, Skin irritancy and Rodent Carcinogenicity. The observations from these studies are tabulated in Table 3. On analyzing these results it can be observed that while most of the analogues show aerobic biodegradability, Analogue 9 shows resistance towards

biodegradation. All the analogues are evidently non-mutagen and non- carcinogen in nature, while a few mild levels of skin irritation was observed.

Table 3. Toxicity profile of various selected designed analogues.

Inhibitor	Aerobic biodegradability	AMES mutagenicity	Skin irritancy	Rodent Carcinogenicity
Derrubone	Degradable	Non-Mutagen	Non-Irritant	Non-Carcinogen
Analogue 1	Degradable	Non-Mutagen	Non-Irritant	Non-Carcinogen
Analogue 2	Degradable	Non-Mutagen	Non-Irritant	Non-Carcinogen
Analogue 3	Degradable	Non-Mutagen	Irritant	Non-Carcinogen
Analogue 6	Degradable	Non-Mutagen	Irritant	Non-Carcinogen
Analogue 7	Degradable	Non-Mutagen	Non-Irritant	Non-Carcinogen
Analogue 8	Degradable	Non-Mutagen	Irritant	Non-Carcinogen
Analogue 9	Non-Degradable	Non-Mutagen	Non-Irritant	Non-Carcinogen
Analogue 10	Degradable	Non-Mutagen	Non-Irritant	Non-Carcinogen
Analogue 26	Degradable	Non-mutagen	Non-Irritant	Non-Carcinogen

4.5 Validation of recognized ATP binding sites and comparison of our designed Derrubone analogues with the analogues synthesized experimentally

Blagg and co-workers performed a set of experiments for the synthesis of Derrubone and related analogues to elucidate the Structure-Activity Relationships through the evaluation against colon and breast cancer cell lines [42]. They also performed anti-proliferative tests on the cell lines and summarized that their two analogues (BDA1 and BDA2 as denoted in this paper) showed better Hsp90 client protein-degradation activity than Derrubone (fig. 9). BDA1 was prepared by transposing its prenyl side chain from 6^{th} to 8^{th} position of the isoflavon core and BDA2 was prepared by substituting with dichlorophenyl at 3^{rd} position in the isoflavon backbone. It was also observed from their experiment that these drug candidates showed a decrease in Her2 and Raf levels, as these two proteins depend upon Hsp90 [42].

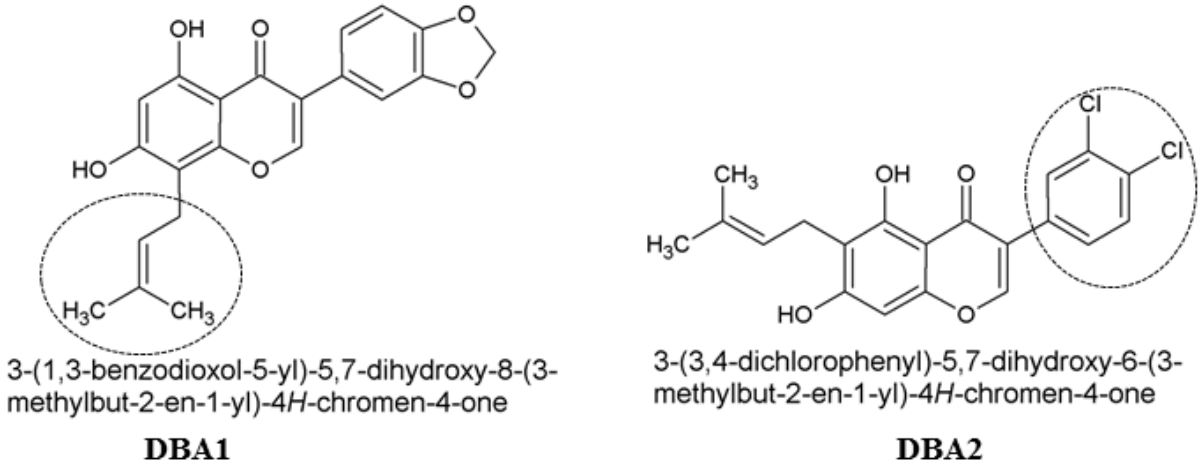


Figure 9. Analogues prepared at Blagg's Laboratory (BDA1 and BDA2)

In our current work, the results obtained by Blagg *et al.* were validated by performing molecular docking experiments and ADME studies of the two best analogues of Derrubone (out of 10 analogues tested) in comparison with Derrubone. The results of TOPKAT studies which are tabulated in Table 4 showed that the AlogP value of BDA1 is the same as that of Derrubone and BDA2 has higher AlogP (>5) which is not in the recommended range. Further, from molecular docking experiments using Autodock 4.0, it was observed that BDA1 (-7.10 kcal/mol) and BDA2 (-7.04 kcal/mol) have better binding affinity than Derrubone (-6.67 kcal/mol) at Leu 694 indicating that these analogues have a higher possibility of binding to this site rather than to other previously recognized sites.ie. Leu 665 and Leu 666 (Table 5). Docking results at Leu 694 also showed that the inhibition constant of both the analogues were found to be upto three times lesser than Derrubone. This result may indicate that Leu 694 may be the natural binding pocket as it is present in the largest cavity of Hsp90α, for Analogue BDA1 and BDA2 given its high binding energy at this site.

It can be validated from these experiments that BDA1 and BDA2 experiences steric hindrance at site Leu 665 and Leu 666, hence, the analogues do not show good binding affinity at these sites as compared to Leu 694. As our recognized ATP binding site i.e. Leu 694 has been

validated from experimental results therefore this site could possibly be the ATP binding site and could be further used to perform molecular docking experiments of our designed analogue to obtain a potent candidate for AD treatment.

Table 4. ADME properties of Blagg analogues.

INHIBITOR	MOLECULAR WEIGHT (<450)	AlogP (<5)	Polar Surface Area (PSA) (<60-90 A^{o2})	ROTATABLE BONDS (<8)	HYDROGEN BOND ACCEPTORS (<7)	HYDROGEN BOND DONORS (<3)
Derrubone	366	4.007	85.122	3	6	2
BDA1	366	4.007	89.130	3	6	2
BDA2	391	5.568	70.700	3	4	2

Table 5. Docking energies and inhibition constant of Blagg analogues against Hsp90*α*.

LIGAND	TARGET	LEUCINE 665		LEUCINE 666		LEUCINE 694	
Drug/ Analogues		Binding energy (kcal/mol)	Inhibition constant (k_i) (µm)	Binding energy (kcal/mol)	Inhibition constant (k_i) (µm)	Binding energy (kcal/mol)	Inhibition constant (k_i) (µm)
Derrubone	Hsp90α CTD	-7.20	5.31	-7.53	2.40	-6.67	12.96
BDA1		-5.04	203.71	-7.35	4.13	-7.10	6.23
BDA2		6.56	15.61	-6.43	19.34	-7.04	6.89

4.6 Molecular Docking of Hsp90α with Derrubone and selective Analogues

The molecular docking studies were carried out using Autodock 4.0 to attain the binding affinitiesof various analogues against Hsp90α CTD. The experimentation was carried out by docking nine previously selected analogues on previously recognized ATP binding residue of

Hsp90α CTD i.e. Leu 694, respectively. The binding energies and the inhibition constant values obtained from the investigation are tabulated in Table 6. The results obtained from the experiment show that Analogue 26 has better binding energy than Derrubone at previously recognized site: Leu 694 (-10.20 kcal/mol) with improved k_i value 0.036 µM.

Table 6. Docking results of Derrubone and designed analogues against Hsp90α.

LIGAND	TARGET	LEUCINE 694	
Drug/ Analogues		Binding energy (kcal/mol)	Inhibition constant (k_i) (µM)
Derrubone	Hsp90α	-6.67	12.96
Analogue 1		-6.65	13.25
Analogue 2		-6.57	15.33
Analogue 3		-5.76	59.65
Analogue 6		-6.51	17.02
Analogue 7		-6.97	7.80
Analogue 8		-6.28	24.95
Analogue 9		-5.25	89.64
Analogue 10		-7.48	3.27
Analogue26		-10.16	0.036

Furthermore, when the results obtained from ADME and toxicity studies and molecular docking experiments of Derrubone, BDA1and BDA2 with Hsp90α CTD, were compared to the results obtained of Analogue 26, it was observed that the AlogP, binding affinity as well as the k_i of Analogue 26 was better than that of Derrubone and Blagg's analogues. The results obtained showed that Analogue 10 could be a potential drug candidate for Hsp90α inhibition as compared to Derrubone, BDA1 and BDA2.

4.7 ADME Plots

As observed from the plot (fig. 10 and fig. 11), it can be seen that Derrubone, BDA1and BDA2 fall beyond the BBB-95 but within BBB-99 ellipse whereas comparatively Analogue 26 falls on the margin of BBB-95 plot. This shows that the former may not be well suited for CNS administration due to their lack of BBB absorption capability. It also shows that Analogue 26 falls into the category of those drugs that are a part of 99% of well-absorbed drugs into the blood brain barrier. It can also be observed from the plot that BDA2 falls marginally on absorption-95 plot indicating that this drug may be less likely to be suited for sufficient intestinal absorption; whereas the remaining three drugs studied show easy intestinal absorption as indicated by their position within absorption-95 and absortion-99 ellipses in the graph.

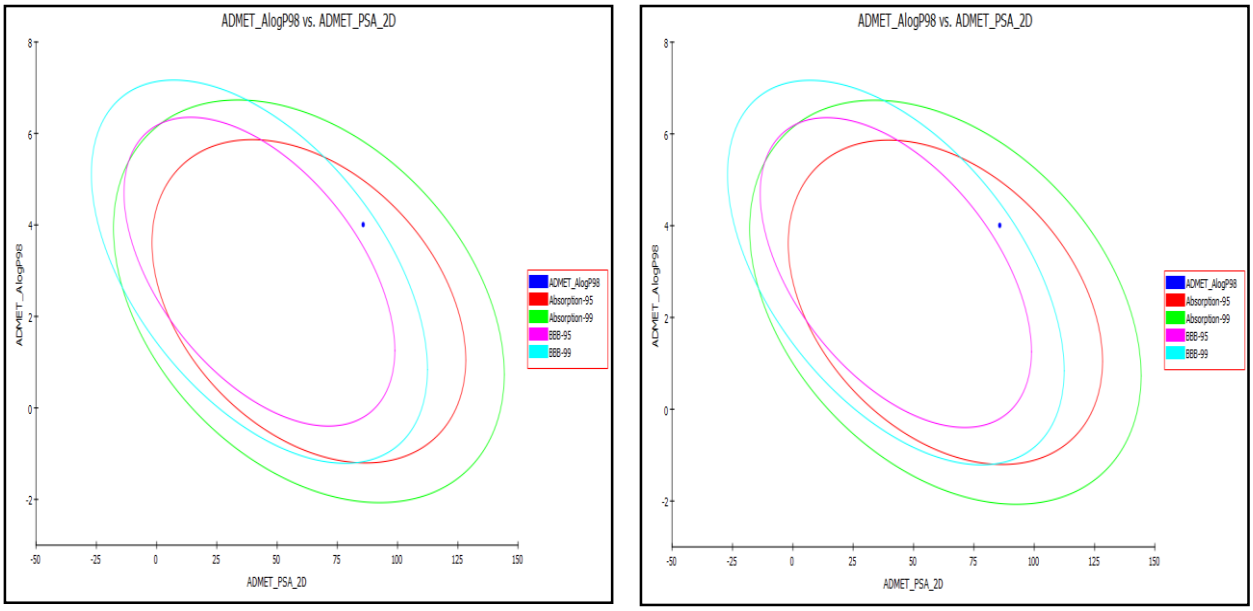


Figure 10. BBB penetration and Human intestinal absorption for: A. Derrubone B. BDA1

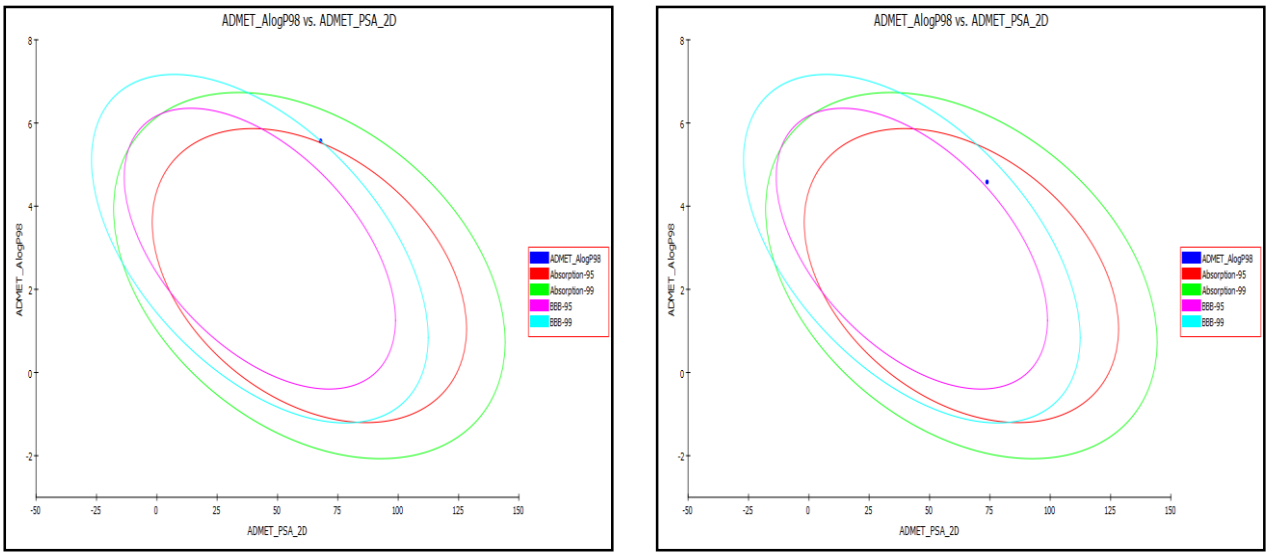


Figure 11. BBB penetration and Human intestinal absorption for: A. BDA2 B. Analogue 26

CHAPTER 4

CONCLUSION

4.1 CONCLUSION

From the present investigation it was concluded that the recognized sites i.e. Leu 665, Leu 667 and Leu 694 could be possible ATP binding sites that are present in the CTD of Hsp90α. The docking experiments which were performed between the various existing drugs and Hsp90α at the three recognized ATP binding sites revealed that Derrubone has the lowest binding energy with minimum value of inhibition constant. It was also observed that at Leu 666 Derrubone gave the best binding affinity (-7.53 kcal/mol) and inhibition constant (2.40μM). The recognition of Derrubone as Hsp90α inhibitor gives a natural product which could be used further for obtaining a more specific novel inhibitor.

To resolve the problems associated with various NTD and CTD blocking drugs including Derrubone, in our present work 30 analogues of Derrubone which can bind to the previously determined ATP binding sites of Hsp90α CTD i.e. Leu 665, Leu 666 and Leu, were prepared. The ADME and toxicity profiles of the existing drugs and analogues were performed using Discovery studio 4.0 to determine a potent inhibitor which has the recommended properties as required for crossing BBB and treating brain maladies such as AD. From the studies it was observed that Analogue 1, Analogue 2, Analogue 3, Analogue 6, Analogue 7, Analogue 8, Analogue 9, Analogue 10 and Analogue 26 have improved lipophilicity as compared to Derrubone and other existing drugs while keeping its molecular weight, number of rotatable bonds, hydrogen bond donor and acceptors within the recommended range as required to be classified as CNS drug. From Extensible Toxicity Protocol studies it was indicated that these analogues are clearly non-mutagen and non- carcinogen in nature, while a few mild levels of skin irritation was observed.

In our present communication, the experimental results obtained from Blagg *et al.* laboratory were validated by performing molecular docking experiments and ADME studies of the two analogues in comparison with Derrubone. The TOPKAT studies showed that the AlogP value of BDA1 is the same as that of Derrubone and BDA2 has higher AlogP (>5) which is not in the recommended range. Further, from molecular docking it was observed that BDA1(-7.10 kcal/mol) and BDA2(-7.04 kcal/mol) have better binding affinity than Derrubone (-6.67 kcal/mol) at Leu 694 of the Hsp-90 CTD, indicating that these analogues have a higher possibility of binding to this site rather than to other previously recognized sites i.e. Leu 665 and Leu 666. Docking results at Leu 694 also showed that the inhibition constant of both the analogues were found to be three times lesser than Derrubone. It can be concluded from this result that Leu 694 may be the natural binding pocket for BDA1 and BDA2 given its high binding energy at this site. Hence, our previously determined ATP binding site Leu 694 had been validated from the experimental results. However, since BDA1and BDA2 were originally tested upon cancer cell lines and the properties of these do not closely resemble to CNS drugs it would be irrelevant to further analyse them for the possibilities of AD treatment.

Further, the study was carried out by docking nine previously selected analogues with previously recognized and validated ATP binding residue Leu 694 of Hsp90α CTD indicated that Analogue 26 (-10.20 kcal/mol) has better binding energy than Derrubone with improved k_i value 0.036 µM. It can be concluded from the ADME graph plotted between AlogP98 vs PSA that Derrubone, BDA1and BDA2 fall beyond the BBB-95 but within BBB-99 ellipse, whereas Analogue 26 falls on the margin of BBB-95 plot. This indicates that the former may not be well suited for CNS administration due to their lack of BBB absorption capability. It also reveals that Analogue 26 falls into the category of those drugs that are a part of 99% of well-absorbed drugs into the blood brain barrier. It can be concluded that the Analogue 26 may be well suited for CNS administration due to its BBB absorption capability. As it was also

observed from the plot that BDA2 falls marginally on absorption-95 plot indicating that this drug may only be marginally suited for intestinal absorption in a small quantity. The remaining three drugs studied show easy intestinal absorption as indicated by their position within absorption-95 and absortion-99 ellipses in the graph. Hence, it can be concluded that Analogue 26 could possibly be used for performing in-vitro and in-vivo experiments for its approval as a potent AD treatment drug. The establishment of ATP binding site in CTD and determining the best inhibitor for Hsp90α could further lead to the effective blockage of Hsp90α which would subsequently result in the degradation of *Tau* proteins for the treatment of Alzheimer's disease.

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Publications

- **Khalid S and Paul S. Identifying C-terminal ATP binding sites-based novel Hsp90-Inhibitor in-silico: A plausible therapeutic approach in Alzheimer's disease. Medical Hypotheses 2014; 83(1): 39-46.**

- **In silico Design of novel Derrubone analogues Hsp90-inhibitor for *Tau* protein degradation: A therapeutic approach in Alzheimer's disease. (Under preparation)**